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HAPLOTYPING OF APOLIPOPROTEIN B GENE BY POLYMERASE CHAIN REACTIONS: IT'S RELATIONSHIP TO SERUM LIPID LEVELS AMONG GERIATRIC CHINESE IN HONG KONG

BY

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on

22nd March, 1994

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Thesis Submitted To

Faculty of Medicine

Division of Clinical And Pathology Sciences

In Fulfilment of The Requirements

For

The Master of Science In Clinical Biochemistry

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ACKNOWLEDGMENTS

I would like to express my sincere thanks to my supervisor, Dr. C. P. Pang, Senior Lecturer, Department of Chemical Pathology, the Chinese University of Hong Kong, for introducing me into this research topic and for his continuous guidance and encouragement.

I would like to devote my thanks to Mr. Y. T. Mak for guiding me in my practical work.

I would also like to express my sincere gratitude to Professor J.R.L. Masarei, Head of Department of Chemical Pathology Laboratory, the Chinese University of Hong Kong, for supporting my study of this MSc. course.

I would also like to thank Dr. Jean Woo who supports this work in helping me to recruit study subjects.

Finally, thanks to Ms. Lisa Chan, Medical Laboratory Technician I in Department of Chemical Laboratory, the Chinese University of Hong Kong for helping DNA extraction and oligonucleotide synthesis, Ms Priscilla Poon, Senior Medical Laboratory Technologist, and Ms. Vicky Lai for sharing with me their experiences in molecular biology techniques.

SUMMARY

Three polymorphic sites of the apolipoprotien B gene in Chinese geriatric subjects were studied by polymerase chain reactions. They were the signal peptide insertion/deletion allele (Leu-Ala-Leu -16/-14), the XbaI site in exon 26 (Thr 2488) and the EcoRI site in exon 29 (Glu 4154/Lys). Cord blood samples from deliveries of normal Chinese infants were used to establish the allelic frequencies at these sites of the Chinese population. The study subjects, age range 57-87, were divided into the case group, having total serum cholesterol > 6.2 mmoL/L and LDL cholesterol > 4.1 mmoL/L, and the control group, total cholesterol < 6.2 mmoL/L and the LDL cholesterol < 4.1 mmoL/L. In the cord blood DNA analysis, there was no sex difference in the frequencies of the rare alleles: the D allele (deletion allele), the X+ allele (presence of the XbaI site) and E- allele (absence of the EcoRI site). The results were consistent with published data on Chinese. There was significant difference between the cord blood samples and the control subjects in frequencies of the D allele, 0.181 vs 0.312, and the E- allele, 0.061 vs 0.413. While the cord blood samples were different with the case subjects only in the D frequency, 0.181 vs 0.267. The D and the E- frequencies were unexpectedly higher in the controls than the cases, 0.312 vs 0.267 and 0.413 vs 0.067 respectively. The pattern of the frequency distribution of the genotype of the insertion/deletion region (II, ID, DD) and of the Xba I site (X-X-, X-X+, X+X+) were similar between the controls and the cord blood samples, but were different with the case subjects. The

genotypic pattern of the EcoRI site (E+E+, E+E-, E-E-) was different between the control and the cord blood samples, but similar between the latter and the case subjects. The only X+ allele found was in two X-X+ control subjects. There were no X+X+ genotype detected in both controls and cases. Presence of the D allele did not correlate with an increase in serum lipid levels in both control and case subjects. Case subjects having the E+E- genotype had higher total serum cholesterol, triglyceride and LDL-cholesterol but lower HDL-cholesterol than those case subjects having the E+E+ genotype. In the control subjects, such relationships were reversed. The view that presence of the D and the X+ alleles was associated with increase in serum cholesterol levels was not affirmed by the results of this study. The conflicting effects of the E- allele on the lipid levels in the controls and cases cast a question on a positive relationship between E- and serum cholesterol. It is possible that the genotypic pattern of these three polymorphic sites of the apolipoprotein B gene and their effects on serum lipid levels are unique among Chinese geriatric subjects. This study also suggested that cord blood samples can be used to obtain reference genotypic patterns of the apolipoprotein B gene in a population.

1. INTRODUCTION

Coronary heart disease (CHD) is a major public health problem in many industrialized countries. Although it is a multifactorial disorder, a number of important contributing factors have been identified over the last 20 years. For example, the association between serum cholesterol and CHD has well been established [1, 2, 6]. Other than environmental factors such as smoking and alcohol intake, genetic variations play an important role in leading to hypercholesterolaemia.

1.1 LIPID METABOLISM

The major lipids present in the plasma are fatty acid, triglyceride, cholesterol and phospholipid. Cholesterol and triglyceride are hydrophobic compounds whereas phospholipid is amphipathic, they are integral components of lipoprotein and cell membrane. Because lipids are water in-soluble, they are transported in blood in association with proteins, for example cholesterol and triglyceride circulate in complexes known as lipoproteins. These consist of an non-polar core of triglyceride and cholesterol ester surrounded by phospholipid, cholesterol and protein known as apolipoprotein. Apolipoprotein is important both structurally and in the metabolism of lipoprotein.

Lipoproteins are different in the floatation rate, hydrated density, size and electrophoretic mobility. Most often they are

separated by ultracentrifugation in the order of increasing density, they are chylomicron (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL).

1.1.1 CHYLOMICRON

Chylomicron consists mainly of triglyceride and major apolipoproteins include apo B-48, apo C and apo E. It transports dietary triglyceride (exogenous triglyceride) from intestine to sites of utilization. The triglyceride of chylomicron is hydrolysed by lipoprotein lipase, located on the luminal surface of the capillary endothelium of adipose tissue, skeletal and cardiac muscle, free fatty acids are then delivered to these tissues either to be used as energy substrates or, after re-esterification to triglyceride, for storage. While the residual particle, called chylomicron remnant, is removed from the plasma rapidly in the liver by a specific receptor which has not yet been fully characterized. Under normal conditions, the above process proceeds rapidly postprandially.

1.1.2 VERY LOW DENSITY LIPOPROTEIN

Endogenous triglyceride, synthesized in the liver, is transported in VLDL which contains cholesterol, triglyceride, apo B-100, apo E and apo C. As the surface components are removed, triglyceride is hydrolysed by lipoprotein lipase, VLDL is then

degraded into IDL. Under normal condition, most IDL particles are removed from blood by binding to the LDL receptor in the liver. IDL particles have a very high affinity for LDL receptor, therefore they have a short lifespan of only a few minutes in blood. A fraction of IDL particles escapes hepatic uptake and undergoes further lipolysis to generate LDL.

1.1.3 LOW DENSITY LIPOPROTEIN

LDL is the main cholesterol carrying particle in plasma. It transports cholesterol from the liver to peripheral tissues. LDL receptor is also called B/E receptor due to its capability of recognizing both apo B and E [1]. LDL can bind to specific LDL receptor on cell surface that recognize apo B-100, the sole protein component of LDL. Subsequently, LDL is internalized and after lysosomal degradation, free cholesterol is then released. When the intracellular cholesterol concentration is increased, the activity of hydroxymethylglutarate-Co A (HMG Co-A) reductase, the rate limiting enzyme of cholesterol synthesis pathway is decreased, the activity of the acyl Co-A cholesterol acyltransferase (ACAT) is increased and the LDL receptor is down regulated.

Cholesterol can also be uptaken by cells through non-receptor-mediated pathway. This may account for two-thirds of cholesterol uptake at a 'normal' serum cholesterol and even more at higher levels. This uncontrolled uptake of cholesterol is thought to be

an important factor in the pathogenesis of atherosclerosis. When macrophages become overloaded with cholesterol esters, they are converted to 'foam cells', a major component of atheromatous plaque.

1.1.4 HIGH DENSITY LIPOPROTEIN

High density lipoprotein, comprising phospholipid, cholesterol, apo AI, apo AII and apo E as main apolipoproteins are synthesized primarily in the liver and, to a lesser extent, in small intestinal cells. HDL have an important function in removing cholesterol from the circulation by assimilation from cellular membrane or through high-density lipoprotein receptor [7, 8, 9, 10]. Epidemiologic studies have shown a correlation between low HDL level and an increased risk of CHD.

1.2 APOLIPOPROTEIN B

The metabolisms of plasma lipoproteins are directed by specific proteins, called apolipoproteins. Two apolipoproteins, apo E and apo B, are ligands that bind LDL receptors on surface of various cells.

Two forms of apo B exist in the plasma : apo B-48 and apo B-100. Apo B-48, a truncated form of approximate 2152 amino acids from the amino terminus, is the structural protein moiety of chylomicron and its remnant. It is synthesized and secreted with

chylomicron in the intestine. It is used for the assembly of chylomicron and has an obligatory role in the absorption of dietary fats. Due to the rapid turnover of the chylomicron, the concentration of apo B-48 in plasma is normally very low. Apo B-100 is the full molecule, a single polypeptide composed of 4536 amino acids, with a molecular weight of about 513 kilo-Daltons (kD). Only apo B-100 possesses receptor binding activity and is required for the assembly of triglyceride rich VLDL particles in the liver. It is also found in IDL and LDL, which are metabolic products of VLDL. Majority of the plasma apo B is in the LDL fraction [1].

1.3 APOLIPOPROTEIN B GENE

The apolipoprotein B gene resides on the short arm of chromosome 2 (2p 23-24) [6]. The complete nucleotide sequence for the apo B cDNA and the deduced amino acid sequence are reported [5].

Apo B gene spans over 43 kb and comprises 29 exons and 28 introns. Exons 26 and 29 are extremely large, 7572 and 1906 bp respectively. The apo B-100 mRNA which is 14.1 kb in length coding for a 24-29 amino acid signal peptide and a 4536 amino acid mature protein [11]. Apo B-48 is encoded by the same apo B gene, however, the mRNA is edited postranscriptionally by a tissue specific enzyme [12] which causes a C-U change of codon CAA specifying 2153 Gln to a stop codon. Therefore, the apo B-48 protein comprises 2152 amino acids from the amino terminal [14].

This process occurs mainly in the intestine and to a lesser extent in the liver [14].

The factors which regulate apo B synthesis and secretion rate are not clear. In intestinal and liver cells DNA methylation has been suggested to participate in the regulation of apo B gene expression.

1.4 GENETIC VARIATIONS IN HUMAN APO B GENE AND THEIR ASSOCIATIONS WITH ABNORMAL LIPID METABOLISM

The main function of the apo B-100 is binding to the LDL receptor for cellular catabolism of LDL, genetic variation of the apo B gene might affect LDL metabolism.

1.4.1 ABETALIPOPROTEINEMIA

Abetalipoproteinemia is a rare autosomal recessive disorder. The disorder is characterized by low levels of plasma cholesterol and triglyceride and complete absence of plasma apo B, VLDL, LDL and chylomicron. In abetalipoproteinemia all other apolipoproteins are found in the HDL fraction. Abetalipoproteinemia is resulted from apo B gene mutations that prevent synthesis of apo B or produce an aberrant apo B mRNA or protein molecule [14].

1.4.2 HYPOBETALIPOPROTEINEMIA

Patients homozygous for hypobetalipoproteinemia have been described lack of plasma apo B, VLDL, LDL and chylomicron. They are phenotypically indistinguishable from patients with abetalipoproteinemia, the only difference appears to be that parents of these patients have half-normal LDL level, whereas parents of abetalipoproteinemic patients have normal LDL level.

1.4.3 FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

Familial defective apo B-100 is a genetic disorder characterized by elevated plasma LDL cholesterol concentration, and in some cases by premature atherosclerosis [15]. DNA sequence analysis of the defective allele has shown a CGG-CAG transition at the codon specifying amino acid 3500, resulting a Gln-Arg substitution [1]. This mutation affects the LDL-LDL receptor interaction.

1.5 POLYMORPHISMS OF APO B GENE

A number of genetic polymorphisms of apo B gene have been reported. [4, 6, 16-20, 32]. They include: (1) insertion/deletion of signal peptide in exon 1, (2) XbaI in exon 26, (3) EcoRI in exon 29, , (4) MspI in promotor site, (5) MspI at codon 3611, (6) StylI in intron 2 and (7) 3′ variable number of tandem repeat. Some of these polymorphisms have been found to be associated with

serum lipid levels [4], peripheral arterial disease [21], coronary artery disease [22, 23] and risk of myocardial infarction [24]. However conflicting results were often obtained by different studies [16-20, 22, 24].

Among various polymorphisms, the following three were well studied: insertion/deletion (I/D) polymorphism of the signal peptide in exon 1, XbaI polymorphism (Thr 2488 C to T) in exon 26 and EcoRI polymorphism (Glu/Lys 4154) in exon 29.

In exon 1, the deletion allele codes for 24 amino acids, when compared with the insertion allele codes for 27 amino acids, three amino acids, leu-ala-leu are deleted. The absence of these amino acids might alter the hydrophobicity of the signal peptide and the rate of translocation of apo B from the cytoplasm into the endoplasmic reticulum, then affecting the rate of apo B synthesis and secretion.

XbaI polymorphism in exon 26 has been found to be associated with variation of cholesterol levels in normal individuals [26-28] and patients with familial hypercholesterolaemia [29]. However the underlying mechanism is unknown. Associations between the EcoRI polymorphism in exon 29 and coronary heart disease have also been reported [22-24,30].

1.6 METHODS FOR DETECTION OF POLYMORPHISMS

Polymerase chain reaction (PCR) is widely used for the amplification of a DNA segment. Polymorphism can be detected by various methods with the PCR product. Small deletion and insertion at defined location can be detected by size difference of the amplified DNA fragments by gel electrophoresis. If the polymorphism creates or abolishes a restriction enzyme cutting site, it can be identified by digestion of the amplified DNA fragments with the appropriate enzyme and then be analyzed by gel electrophoresis.

In the setting up of each PCR protocol, some factors that would affect the amplification reaction should be evaluated. These include:

(1) Effect of magnesium chloride concentration in the reaction mixture.

(2) Annealing temperature should be optimized. It is related to the melting temperature (T_m) of the pair of primers used. Melting temperature is estimated from the number of adenosine (A), thymine (T), cytosine (C) and guanine (G) in each DNA primer (T_m = [2 X (A + T) + 4 X (C + G)]°C. The annealing temperature should be 4 to 5°C below the T_m.

(3) Period of incubation in each step of the temperature program.

2. OBJECTIVES

1. To set up a polymerase chain reaction for the detection of three polymorphisms of the apo B gene : signal peptide insertion/deletion polymorphism in exon 1, XbaI in exon 26 and EcoRI in exon 29.

2. To determine the allelic distribution frequenies of the three polymorphisms of the apo B gene in cord blood samples and geriatric Chinese in Hong Kong.

3. To correlate the three polymorphisms of the apo B gene and serum lipid levels among geriatric Chinese in Hong Kong.

3. MATERIALS AND METHODS

3.1 MATERIALS AND EQUIPMENTS

3.1.1 ENZYMES

Taq DNA polymerase was obtained from GIBCO-BRL, Life Technologists, Inc., Gaithersburg, USA. Two restriction enzymes EcoRI (20,000 U/mL) and XbaI (20,000 U/mL) were purchased from New England Biolabs, Beverly, USA.

3.1.2 DNA MARKERS

HaeIII digested pBR322 DNA was purchased from Sigma Chemical Co., St. Louis, USA. 1 Kb lambda DNA was obtained from GIBCO-BRL, Life Technologies, Inc., Gaithersburg, USA.

3.1.3 GENERAL REAGENTS

All reagents used were either of biochemical or AR grade. Agarose, dimethyl sulphoxide and ethidium bromide were obtained from Sigma Chemical Co., St Louis, USA. Bromophenol blue and paraffin liquid were purchased from E. Merk, Darmstadt, Germany.

3.1.4 EQUIPMENTS

Perkin Elmer Cetus Thermal cycler 480 was purchased from Perkin

Elmer Co., Norwalk, USA. Electrophoresis tank, model horizon 11.14 was bought from GIBCO-BRL, Life Technologies, Inc., Gaithersburg, USA. UV-Transilluminator model TS-36 with polaroid camera was purchased from UltraViolet Product, Inc., USA. DNA synthesizer Cyclone Plus was purchased from Millipore Co., Bedford, USA. ACS 180 was purchased from Ciba Corning Diagnostic Cop., Medfield, USA and Dimension AR was purchased from Dupont Medical Products, Wilmington, USA. Cobas Mira was purchased from F. Hoffmann-La Roche & Company, Baole, Switzerland.

3.2 BUFFERS

10 X TAE buffer used was 0.4 M Tris-BASE and 0.001 M EDTA in distilled water and pH of 7.4. 10 X Polmix buffer was 500 mM potassium chloride (KCl), 100 mM Tris-HCl, 0.01 % gelatin and 2 mM each of deoxynucleotide triphosphate (dNTP) in distilled water. Gel loading buffer was 0.25% (v/w) bromophenol blue and 15% (v/v) Ficoll in distilled water.

3.3 AGAROSE GEL ELECTROPHORESIS

Horizontal agarose gel electrophoresis was carried out on a gel electrophoresis tank. Agarose gel was prepared in 1 X TAE buffer. The slurry was heated in a microwave oven until complete dissolution. Ethidium bromide (10 mg/mL) was added to give a final concentration of 0.5 ug/mL. Then the gel was poured into an electrophoresis tray for solidification. Comb was placed

immediately, the teeth of which would form the sample wells. After setting of the gel, the comb was removed carefully and the gel was put in the electrophoresis tank. Enough 1 X TAE buffer was added into the tank to cover the gel surface [31]. DNA marker and samples mixed with loading buffer were loaded into the sample wells of the gel. Electrophoresis was then carried out by running at 50-100 mA until the bromophenol blue migrated to the desired distance on the gel. After electrophoresis, the gel was viewed on the UV transilluminator and photograph taken under UV light with Polaroid film.

3.4 STUDY SUBJECTS

3.4.1 CORD BLOOD SAMPLES

Cord blood samples in EDTA tubes were collected from normal deliveries of Chinese babies in Hong Kong. Genomic DNA was extracted.

3.4.2 GERIATRIC SUBJECTS

CASES

Patients recruited into this group included 12 women and 6 men aged 60-80 with high total cholesterol (> 6.2 mmoL/L) and LDL cholesterol (> 4.1 mmoL/L). Patients fulfilled the selection

criteria with known secondary causes of hyperlipidaemia such as diabetes mellitus and hypothyroidism but well controlled were also included.

CONTROLS

The control group included 18 women and 6 men aged 57-87 with normal serum lipids levels, their total cholesterol and LDL cholesterol were below 6.2 and 4.1 mmoL/L respectively.

Reference intervals of serum lipid are listed in Appendix I.

3.5 CLINICAL DATA

Weight and height of each subject were measured. Body mass index (BMI) was then calculated [Weight (Kg) / Height^2 (m^2)]. History of smoking, alcohol and drug intake were also recorded.

3.6 BLOOD COLLECTION

Fasting blood was collected from each subject by venepuncture into EDTA tube for DNA study, heparin tube for biochemical analysis, plain tube for thyroid function test and fluoride tube for glucose analysis.

The EDTA blood tubes were stored at -20°C for not more than 3 months prior to DNA extraction. Plasma and serum were separated

as soon as possible for biochemical analysis.

3.7 BIOCHEMICAL ANALYSIS

Thyroid function test was performed on ACS 180 (Ciba Corning Diagnostic Cop., Medfield, USA). Renal function test, liver function test, lipid profile, cardiac enzymes, uric acid and gamma glutamyl transferase were performed on Dimension AR (Dupont Medical Products, Wilmington, USA). Plasma glucose was done on Cobas Mira (F. Hoffmann-La Roche & Company, Baole, Switzerland).

All the biochemical tests were carried out according to the manufacturer's procedures. HDL cholesterol was measured using the supernatant after phosphotungstate magnesium precipitation. LDL cholesterol was calculated using the Friedewald formula (LDL cholesterol = Total cholesterol - HDL cholesterol - Triglyceride/2.3).

3.8 DNA EXTRACTIONS

Salting out method [37] was used to extract DNA from thawed EDTA blood.

3.9 POLYMERASE CHAIN REACTION (PCR)

The PCR protocols were modified from reference 20.

OLIGONUCLEOTIDE PRIMERS

Oligonucleotide primers were synthesized on a DNA synthesizer, Cyclone Plus 480 (Millipore Co., Bedford, USA). Sequences of the 5′ upstream and 3′ downstream primers and their corresponging positions on cDNA of the apo B gene were shown in Table 1.

Amplification was carried out in a final volume of 50 ul containing 200 ng of genomic DNA, 250 ug of each primer, 10% dimethyl sulphoxide (DMSO), 5 uL 10 X Polmix buffer and 0.5 units of Taq DNA polymerase. The reaction mixture was topped with 50 uL of liquid paraffin to prevent evaporation.

A water blank with the composition as the sample tube except the genomic DNA was run in parallel to check for contamination.

The amplification was performed on a Perkin Elmer Cetus Thermal Cycler (Perkin Elmer Co., Norwalk, USA.).

SIGNAL PEPTIDE INSERTION/DELETION POLYMORPHISM

In the study of insertion/deletion (I/D) of signal peptide in exon 1, fragments of 93/84 base pairs (bp) were amplified in a

reaction mixture with 1.0 mM $MgCl_2$, under the conditions of 40 cycles of denaturation at 95°C for 1 minute and annealing at 65°C for 1.5 minutes without extension period.

The PCR product (10 uL) was analysed on a 5% agarose gel at a current of 100 mA for 45 minutes. Hae III digested pBR322 was used as marker.

XbaI POLYMORPHISM

The XbaI polymorphism was analyzed by amplifying a 710 bp fragment in a reaction mixture of 3.0 mM $MgCl_2$. The condition for amplification was an initial denaturation period of 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 1.5 minutes, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes. Finally an extra extension period of 5 minutes at 72°C was carried out.

Ten uL of PCR products was checked in 1% agarose gel, run at a current of 100 mA for one hour with 1 Kb lambda as marker. Then overnight digestion of the PCR product with 10 U of XbaI at 37°C resulted in fragments of 433 bp and 277 bp in the presence of cutting site. Electrophoretic separation of 10 uL of the digested PCR products was performed on 2.5% agarose gel at a current of 100 mA for one hour with 1 Kb lambda as marker.

EcoRI POLYMORPHISM

For analysis of the EcoRI polymorphism, a fragment of 480 bp was amplified in a reaction mixture with 1.0 mM $MgCl_2$. The PCR program was carried out by initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1.5 minutes, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes. Finally, an extra extension period of 5 minutes at 72°C was carried out.

Ten uL of amplified product (480 bp) was checked on a 2% agarose gel electrophoreted at a current of 100 mA for one hour using 1Kb lambda as marker. Overnight digestion of the PCR products with 10 U of EcoRI at 37°C gave 253 bp and 227 bp fragments in the presence of the cutting site. Agarose gel electrophoresis of 10 uL of the digested PCR product was then performed in 3% agarose gel at a current of 100 mA for one hour.

3.10 DATA ANALYSIS

The allelic frequencies of the three polymorphisms in the cord blood samples, case and control groups were estimated by gene counting method. The significance of the differences of allelic frequencies between groups was tested by chi-square test. The significance of differences between the lipid levels and demographic data in different groups were estimated by students' t-test. Analysis of variances (ANOVA) with sex, age and BMI as

covariates was performed to estimate the percentage of sample variance (R^2 x 100) of serum lipids which could be explained by each of the apo B gene polymorphisms. Data on ANOVA were not presented as none of the tested polymorphisms reached the required level of significance ($p < 0.05$). All the statistical analysis were carried out using the software Abstat package.

4. RESULTS

4.1 OPTIMIZATION OF PCR

PCR protocols for the three polymorphisms were optimized by studying the magnesium concentration in the PCR buffer and annealing temperature in the PCR temperature program. Results of the investigations on the PCR of the EcoRI and signal peptide insertion/deletion site were shown in Fig. 1 and 2 respectively. The bands with the highest light intensity represented the best PCR production. The results appeared the best at a magnesium concentration of 1.0 mM and an annealing temperature of 65°C on the PCR protocol of the signal peptide insertion/deletion site. In the PCR protocol for studying XbaI polymorphism on exon 26 and Eco RI polymorphism on exon 29, the magnesium concentration was 3.0 and 1.0 mM respectively and annealing temperature were both of 55°C.

4.2 CLINICAL FEATURES OF THE CASE AND CONTROL SUBJECTS

Both groups of subjects had similar proportion of males and females (Table 2). They also have statistically comparable age, height, weight and body mass index (BMI) according to the students' t-test. The case subjects had significantly higher serum levels of total cholesterol (TC), triglyceride (TG), LDL-cholesterol (LDL-C) and lower HDL-cholesterol (HDL-C) than the control subjects (Table 2).

4.3 GENOTYPING

The allelic frequencies of the three polymorphic sites, I and D (the insertion and deletion alleles respectively of the signal peptide insertion/deletion site), X- and X+ (the alleles corresponding to the absence and presence respectively of the Xba I site in exon 26) and E+ and E- (the alleles corresponding to the presence and absence respectively of the Eco RI site of exon 29) obtained in the cord blood samples were consistent with reported values in the literature (Table 3). They generally agreed with the Hardy-Weinberg equilibrium. The only exception was the allelic frequencies of the signal peptide region of the female cord blood samples in that there was small statistical difference ($p < 0.05$, chi-square test) between the actual and the expected frequencies (Table 3). The female and male cord blood samples agreed in their allelic frequencies. Therefore the results of all the cord blood samples were used for comparison with control and case subjects.

Two of the rare alleles, D and E- were found to be of surprisingly high frequencies in the control subjects (Table 4). They were even statistically higher than those of the case subjects, 0.312 vs 0.267 and 0.413 vs 0.067 respectively, and higher than those of the cord blood samples, 0.312 vs 0.181 and 0.413 vs 0.061 respectively. The other rare allele, X+, was infrequent in the control subjects (0.042) and in the cord blood

samples (0.061) and was absent in the case subjects. It is noted that the cord blood samples resemble the case subjects more than the controls in allelic distributions of the three polymorphic sites.

Case subjects having the ID genotype did not show any significant difference in serum levels of any of the lipids from those of the II genotype (Table 5). In the control subjects, the ID genotype did result in lower TC, TG and LDL-C. The DD genotype, however, reversed the effect (Table 5).

No X+ allele was detected in the case subjects and there were two control subjects with the X-X+ genotype (Table 6). These two controls had higher TG and correspondingly lower HDL-C when compared with the other control subjects having the X-X- genotype.

No E-E- genotype was detected in both control and case subjects but the relative frequencies of the E+E+ and E-E+ genotypes were reversed in the two subject groups. The effects of the rare allele E- on the serum lipid levels were conflicting. Presence of E- resulted in higher TC, TG and LDL-C and lower HDL-C in the case subjects but the effects were reversed in the control subjects (Table 7). It is noted that the control subjects having the E+E- genotype actually had lower TC and LDL-C than those having the E+E+ genotype.

5. DISCUSSION

5.1 OPTIMIZATION OF PCR PROTOCOLS

The polymerase chain reaction (PCR) has become a popular research technique, mainly because of its effectiveness and reliability in in vitro amplification. However the success of a PCR depends on stringent reaction conditions. To set up a PCR protocol, an optimization procedure is always required.

Magnesium concentration and temperature program were the two reaction conditions evaluated during the optimization of each PCR protocol in this study. Magnesium concentration of about 1.5 mM with 200 uM each deoxynucleotide triphosphate (dNTP) are commonly used [32], however, in some circumstances, different amount of magnesium may be necessary. In this study, effects of magnesium concentration at 1.0, 2.0, 3.0 and 4.0 mM in PCR buffer were investigated in each PCR protocol. Excess magnesium will result in non-specific amplification while insufficient of which will reduce the yield [32]. A magnesium concentration of 1 mmoL/L was found to be optimal for the two PCR protocols (study of signal peptide insertion/deletion and EcoRI polymorphisms) in this study. But in the PCR protocol for the study of XbaI polymorphism, the optimal concentration was 3.0 mmoL/L.

Temperature program greatly affect the result of a PCR. The denaturation temperature is usually between 90-95°C for complete

denaturation of double stranded DNA. The annealing temperature that affects the annealing of their complementary sequences on the template and therefore affect the specificity and yield of the reaction. Usual annealing temperatures are 4 to 5°C below the melting temperatures of the primers. The annealing temperatures of the three PCR protocols in this study were all below the melting temperature of the corresponding primers. Finally the extension time is related to the length of target DNA fragment to be amplified. The extension step can be eliminated if the target sequence is approximately 150 bases or less [36]. In this study the extension step was omitted in the PCR of the signal peptide insertion/deletion polymorphism of apo B gene, in which the amplified DNA fragments were 84 and 93 bp.

5.2 CLINICAL DATA

Age and BMI are the two important covariants that will influence the investigation of the association of the three polymorphisms of apo B gene with hyperlipidaemia [20]. As there were no significant differences of these two parameters between the two groups and test of ANOVA with these two parameters of the polymorphisms did not reach the required level of significance ($p < 0.05$), the test of associations of the three polymorphisms of apo B with hyperlipidaemia could simply be carried out without considering these covariants.

5.3 ALLELIC FREQUENCIES OF THE THREE POLYMORPHISMS OF APO B GENE

There was no sex difference in the frequencies of the rare alleles: the D allele (deletion allele), the X+ allele (presence of the Xba I site) and E- allele (absence of the Eco RI site). The frequencies of the rare alleles (D at 0.181, X+ at 0.061 and E- at 0.061) were similar to those previously reported in the Chinese control samples [20] (D at 0.207, X+ at 0.091 and E- at 0.071). Therefore, the cord blood samples can be used to obtain reference genotypic patterns of the apolipoprotein B gene in the Chinese population in Hong Kong.

The rare alleles D and E- are known to be associated with high serum lipid levels [6, 20]. However, in this study their frequencies are found to be higher in the control subjects than in the case subjects who were hyperlipidaemic. Although the relatively small number of subjects in this study may not truly reflect the actual relationship, it is possible that the phenotypic expression of those genes may interact with the other genes and the environment to alter the pattern of serum lipid levels. The pattern of the frequency distribution of the genotype of the insertion/deletion region (II, ID, DD) and of the Xb I site (X-X-, X-X+, X+X+) were similar between the controls and the cord blood samples, but were different with the case subjects which indicated that the case subjects might associate with some genetic defects in these two regions. The genotypic pattern of

the EcoRI site (E+E+, E+E-, E-E-) was different between the control and the cord blood samples, but similar between the latter and the case subjects, reason was again unclear. There were no X+X+ genotype detected in both controls and cases, the only X+ allele found was in two X-X+ control subjects because the frequency of the X+ allele in the Chinese is very low.

5.4 ASSOCIATION OF POLYMORPHISMS OF APO B GENE WITH THE CASE GROUP

If an apo B gene polymorphism contribute to the increased level of total cholesterol, triglyceride, LDL cholesterol and decreased level of HDL cholesterol, one would expect a difference in allele frequencies at the apo B gene locus between the case and control groups. However, results of this study showed no significant difference in allele frequencies at the three polymorphic sites (signal peptide ins/del in exon 1, XbaI in exon 26 and EcoRI in exon 29) between the case and control subjects. May be the numbers of subjects in the two groups were too small to achieve significant statistical differences.

5.5 ASSOCIATION OF POLYMORPHISMS OF APO B GENE WITH HYPERLIPIDAEMIA

SIGNAL PEPTIDE INSERTION/DELETION POLYMORPHISM

Signal peptide alterations have two profound effects: decrease

the efficiency of export and alter protein processing [19]. These alterations may affect the encoded information, folding and the hydrophobicity of the leader peptide [26]. Each of them have a role in membrane translocation and consequently on apo B synthesis and secretion. The D allele is characterized by the absence of three amino acids, leu-ala-leu, when compared with the I allele. The absence of these amino acids alters the hydrophobicity of the signal peptide. Therefore, this effect could alter the rate of translocation of apo B from the cytoplasm into the endoplasmic reticulum. Consequently, the rate of secretion of apo B from hepatocytes in the form of LDL would be different, which would directly affect the serum levels of LDL cholesterol [19].

However, presence of the D allele in this study did not correlate with an increase in serum lipid levels in both control and case subjects. But a correlation existed in the Chinese control samples [6], in which D allele correlated strongly with increased level of total cholesterol.

As there is exchange of cholesteryl esters between LDL and HDL particles which is mediated by cholesteryl ester transfer protein (CETP) [36], a mutation in the apo B gene which affected this exchange could lead to lower levels of HDL cholesterol. Therefore, if this second mutation is in linkage disequilibrium with the signal peptide insertion/deletion polymorphism, the association of D allele with low levels of HDL cholesterol found

in the control group could be possible.

XbaI polymorphism

Correlation of the X+ allele of the XbaI polymorphism with higher levels of total cholesterol has been reported in several populations of European origin [22, 26-28]. In this study, the X+ allele was found to correlate with high levels of triglyceride and in particular low levels of HDL cholesterol only in the control subjects. This corresponds with a study of association of apolipoprotein B gene polymorphisms with lipid levels in men of South Asian descent [20]. The mutation that creates the XbaI restriction site does not create a change in the amino acid residue at 2488, so it is unlikely to affect lipid metabolism directly. The XbaI polymorphism was therefore presumed to be a marker in linkage disequilibrium with a functional mutation in the apo B gene itself or in the neighbouring sequence that affects cholesterol levels.

The correlation of X+ allele with low level of HDL cholesterol could be explained by second mutation which is in linkage disequilibrium with the XbaI polymorphism. The most common mutation found was that affect the exchange of cholesteryl ester with LDL and HDL particles mediated by CETP as described before in the signal peptide insertion/deletion polymorphism.

EcoRI polymorphism

Case subjects having the E+E- genotype had higher total serum cholesterol, triglyceride and LDL-cholesterol but lower HDL-cholesterol than those case subjects having the E+E+ genotype. In the control subjects, such relationships were reversed. Actually many studies revealed a positive correlation of E-allele with coronary artery disease only but not serum lipid levels [6, 16, 20, 22-24].

5.6 CONCLUSION

There was no conclusive correlation of apo B polymorphism with serum lipids levels. The view that presence of the D and the X+ alleles was associated with increase in serum cholesterol levels was not affirmed by the results of this study. The conflicting effects of the E- allele on the lipid levels in the controls and cases cast a question on a positive relationship between E- and serum cholesterol.

A number of explanations for the lack of consistency in the association between the three apo B gene polymorphisms with serum lipid levels among the two study subjects could be suggested.

Firstly, lipid metabolism is determined by more than one gene. Interactions between these genes can differ between individuals of the same population. Secondly, phenotypic expression of those genes may depend on interactions between the genes and the environment, which may result in one allele being associated with

hyperlipidaemia in one environment and other allele of the same gene associated with hyperlipidaemia in another environment. Thirdly, influences of factors such as diet and exercise on serum lipid levels of each individual could be important. Lastly, the sample size of both groups was small (case : 15, control : 24) that may not be able to achieve significant differences statistically. In addition, the frequencies of the rare alleles (D, X+ and E-) in the Chinese population are low, it was very difficult to reveal true and weak association between them with serum lipids levels.

Other than the signal peptide insertion/deletion, XbaI and EcoRI polymorphisms, there are some other known polymorphisms in apo B gene may associate with coronay heart disease or lipid levels. More studies enclosing more polymorphisms together with much larger sample size may be useful in understanding the full picture of association of apo B gene polymorphisms with serum lipid levels.

Fig. 1 : Effect of magnesium concentration on the PCR of EcoRI polymorphism of apo B gene of 4 cord blood samples.

Lane	**1**	: pBR322 DNA marker
Lane	**2 - 5**	: Sample 1
Lane	**6 - 9**	: Sample 2
Lane	**10 - 13**	: Sample 3
Lane	**14 - 17**	: Sample 4
Lane	**18**	: water control
Lane	**19**	: 1 Kb lambda DNA marker

Lane	**2, 6, 10 and 14**	: 1.0 mM $MgCl_2$
Lane	**3, 7, 11 and 15**	: 2.0 mM $MgCl_2$
Lane	**4, 8, 12 and 16**	: 3.0 mM $MgCl_2$
Lane	**5, 9, 13 and 17**	: 4.0 mM $MgCl_2$

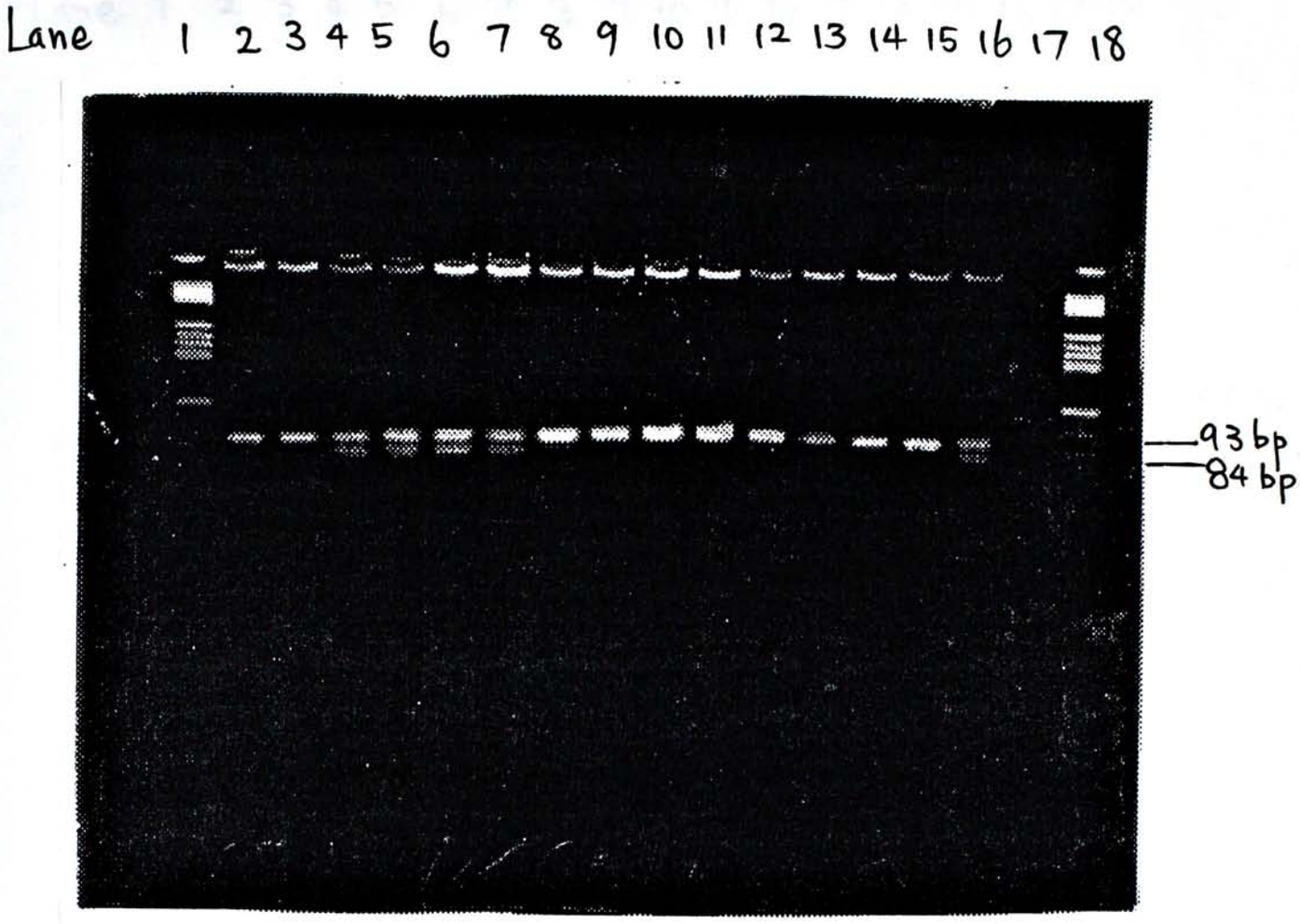

Fig. 2 : PCR of insertion/deletion polymorphism of signal peptide of apo B gene of 15 cord blood samples under correct annealing temperature (65°C)

Lane	**1, 18**	:	pBR322 DNA marker
Lane	**2 - 16**	:	Cord blood samples
Lane	**17**	:	water control

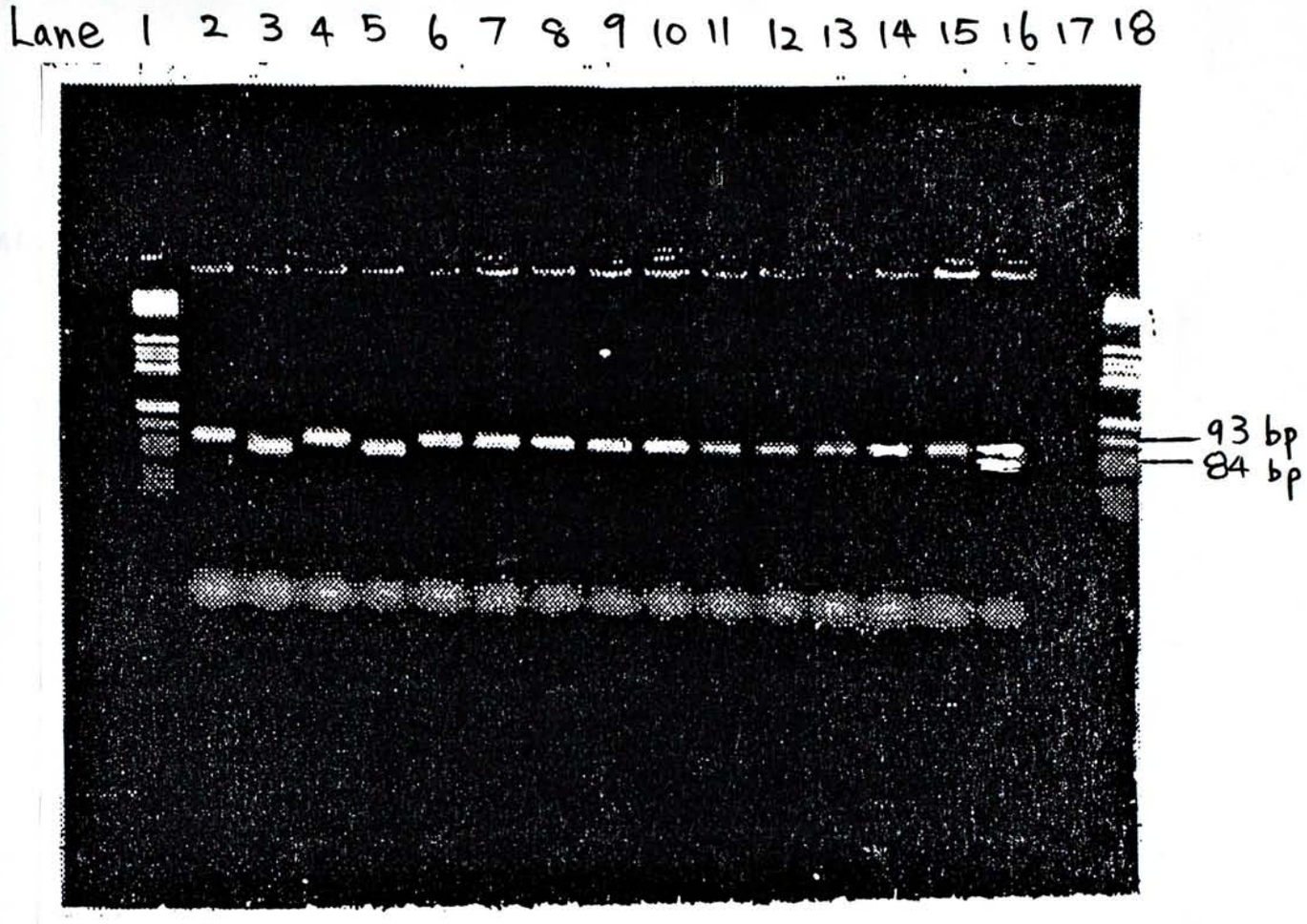

Fig. 3 : Gel electrophoresis of 15 cord blood samples to illustrate the genotypes at the signal peptide insertion/deletion (I/D) polymorphic site in exon 1 of apo B gene. Size of PCR products were given in base pairs.

Lane 1, 18	:	pBR322 DNA marker
Lane 2, 4, 6-15	:	II genotype
Lane 3, 5	:	DD genotype
Lane 16	:	ID genotype
Lane 17	:	water control

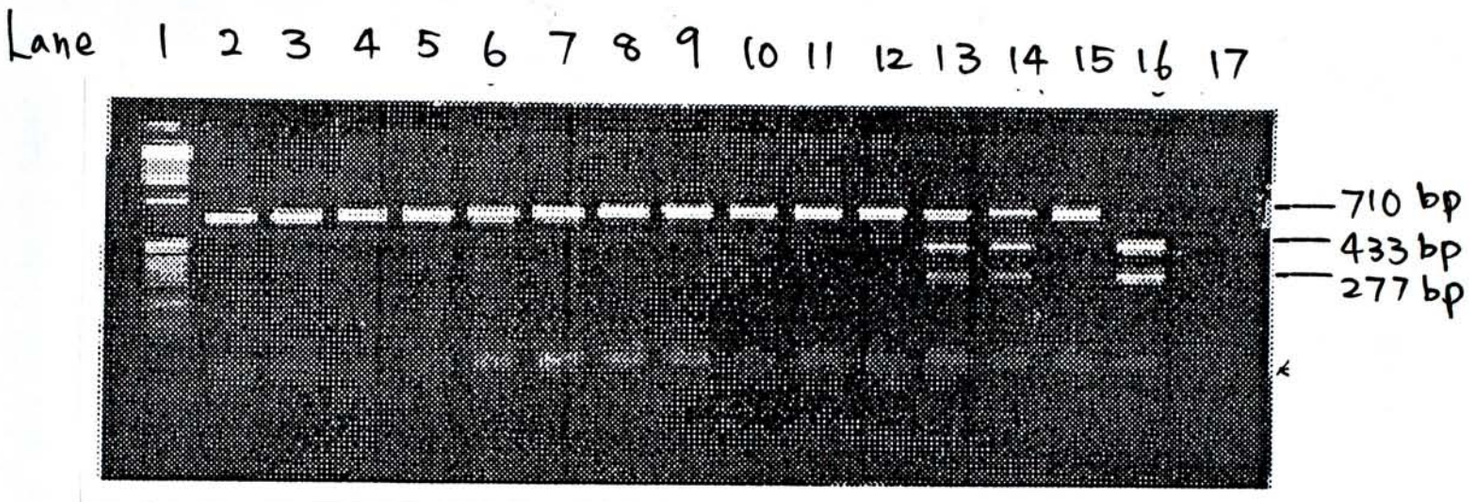

Fig. 4 : Gel electrophoresis of 15 cord blood samples to illustrate genotypes at the XbaI polymorphic site in exon 26 of apo B gene [+, - referring to presence, absence of cutting site for restriction enzyme Xba I (X)]. Size of PCR products were given in base pairs.

Lane	**1**	: 1Kb lambda DNA marker
Lane	**2-12, 15**	: X-/X- genotype
Lane	**13, 14**	: X+/X- genotype
Lane	**16**	: X+/X+ genotype
Lane	**17**	: water control

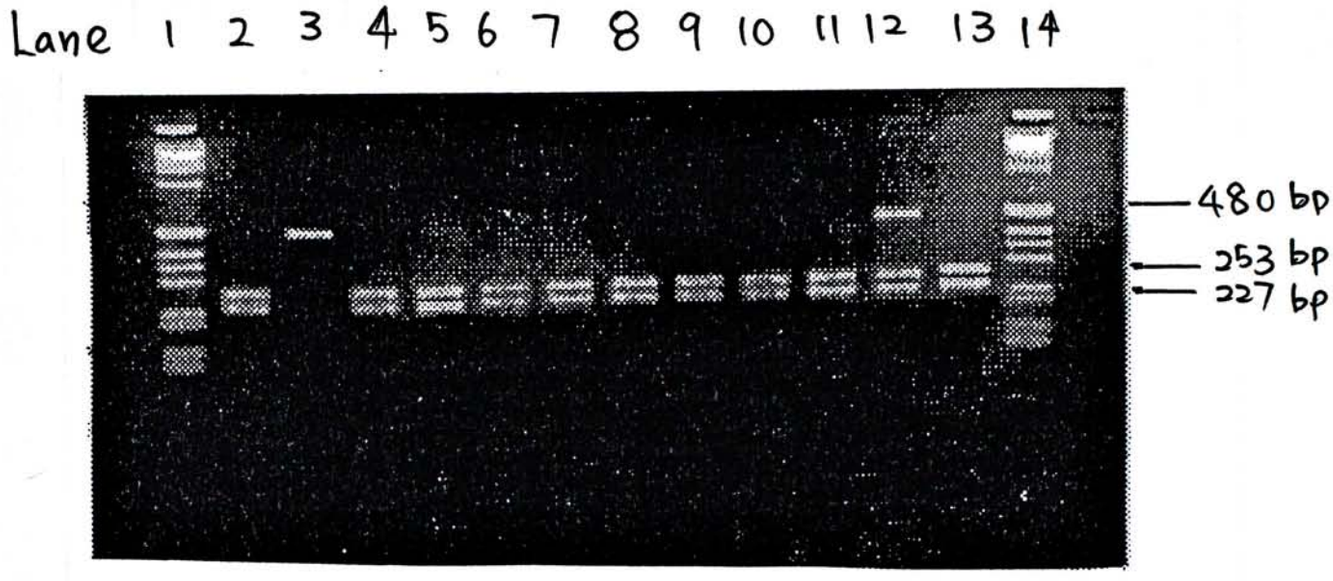

Fig. 5 : Gel electrophoresis of 12 cord blood samples to illustrate genotypes at the EcoRI polymorphic site in exon 29 of apo B gene [+, - referring to presence, absence of cutting site for restriction enzyme Eco RI (E)]. Size of PCR products were given in base pairs.

Lane 1, 14	:	1Kb lambda DNA marker
Lane 2, 4-11, 13	:	E+/E+ genotype
Lane 3	:	E-/E- genotype
Lane 12	:	E+/E- genotype

Table 1: PCR primers used for amplification of the three interested polymorphic sites and their corresponding positions in the apo B gene

Polymorphism	Primer #	Indentity	5' position in cDNA	Sequence	3' position in cDNA
Signal peptide insertion/ deletion	016	5' upstream	1017	5'- CAG CTG GCG ATG GAC CCG CCG A - 3'	1038
	021	3' downstream	1088	5'- ACC GGC CCT GGC GCC CGC CAG CA - 3'	1110
XbaI	024	5' upstream	23916	5'- GGA GAC TAT TCA GAA GCT AAG - 3'	23936
	020	3' downstream	24376	5'- GAA GAG CCT GAA GAC TGA CT - 3'	24395
EcoRI	018	5' upstream	17293	5'- CTG AGA GAA GTG TCT TCA AAG - 3'	17313
	019	3' downstream	17983	5'- CTC GAA AGG AAG TGT AAT CAC - 3'	18003

Table 2 : Clinical features of case and control Chinese geriatric subjects [Mean(SD)]

		Case	Control
	Number of subjects	15	24
	Male / Female	3 / 12	6 / 18
	Age (years)	69.3 (6.70)	69.2 (8.52)
	Height(cm)	152.3 (8.02)	154.9 (9.90)
	Weight (Kg)	56.6 (11.31)	57.9 (10.88)
	BMI (Kg/m^2)	24.3 (3.29)	23.6 (3.92)
#	TC (mmoL/L)	7.97 (0.74)	5.55 (1.08)
#	TG (mmoL/L)	2.49 (1.34)	1.56 (0.98)
*	HDL-C (mmoL/L)	1.23 (0.35)	1.39 (0.59)
#	LDL-C (mmoL/L)	5.84 (0.85)	3.35 (1.22)

* $p < 0.05$, # $p < 0.01$ by students' t-test.

Abbreviations- BMI: Body Mass Index, TC: Total cholesterol, TG: Triglyceride, LDL-C: LDL cholesterol, HDL-C: HDL cholesterol, NS: not significant.

Table 3 : Frequency distributions of polymorphisms : signal peptide insertion/deletion (I/D), XbaI and EcoRI polymorphisms in apo B gene of the cord blood samples.

RFLP	Genotype	Female O	Female E	Male O	Male E	Total O	Total E
Signal Peptide insertion/ deletion	II	* 41 *	(38.7)	39	(39.3)	80	(77.8)
	ID	* 13 *	(18.2)	17	(16.1)	30	(34.4)
	DD	* 5 *	(2.1)	1	(1.6)	6	(3.8)
	Total	59	(59.0)	57	(57.0)	116	(116.0)
Allelic Frequency	I	0.810		0.830		0.819	
	D	0.190		0.170		0.181	
Xba I	X-X-	51	(50.3)	52	(52.1)	103	(102.4)
	X-X+	7	(8.4)	5	(4.8)	12	(13.2)
	X+X+	1	(0.3)	0	(0.1)	1	(0.4)
	Total	59	(59.0)	57	(57.0)	116	(116.0)
Allelic Frequency	X-	0.924		0.956		0.939	
	X+	0.076		0.044		0.061	
Eco RI	E+E+	51	(51.1)	52	(51.3)	103	(106.5)
	E+E-	8	(7.7)	4	(5.3)	12	(9.1)
	E-E-	0	(0.2)	1	(0.4)	1	(0.4)
	Total	59	(59.0)	57	(57.0)	116	(116.0)
Allelic Frequency	E+	0.932		0.947		0.939	
	E-	0.068		0.053		0.061	

* $p < 0.05$, compare significance of difference between observed and expected number of samples by chi-square test.

Abbreviations- O: observed number of samples, E: expected number of samples, I: signal peptide insertion allele, D: signal peptide deletion allele, +: presence of cutting site for specific restriction enzyme, -: absence of cutting site for specific restriction enzyme, E: Eco RI, X: Xba I, RFLP: restriction fragment length polymorphism.

Table 4 : Frequency distributions of polymorphisms : signal peptide insertion/deletion (I/D), XbaI and EcoRI polymorphisms in apo B gene of the case and control Chinese geriatric subjects

RFLP	Genotype	Case O	Case E	Control O	Control E	p
Signal Peptide insertion/ deletion	II	7	(8.0)	12	(11.4)	
	ID	8	(5.9)	9	(10.2)	
	DD	0	(1.1)	3	(2.4)	
	Total	15	(15.0)	24	(24.0)	
Allelic Frequencies	I		0.733		0.688	NS
	D		0.267		0.312	
XbaI	X-X-	15	(15.0)	22	(22.0)	
	X-X+	0	(0.0)	2	(2.0)	
	X+X+	0	(0.0)	0	(0.0)	
	Total	15	(15.0)	24	(24.0)	
Allelic Frequencies	X-		1.000		0.958	NS
	X+		0.000		0.042	
EcoRI	E+E+	13	(13.0)	4	(7.9)	
	E+E-	2	(2.0)	19	(11.2)	
	E-E-	0	(0.0)	0	(0.0)	
	Total	15	(15.0)	23	(23.0)	
Allelic Frequencies	E+		0.933		0.587	NS
	E-		0.067		0.413	

p value for significance of difference of allele frequencies between case and control geriatric Chinese by chi-square test.

Abbreviations- O: observed number of samples, E : expected number of samples, I: signal peptide insertion allele, D: signal peptide deletion allele, +: presence of cutting site for specific restriction enzyme, -: absence of cutting site for specific restriction enzyme, E: EcoRI, X: XbaI, RFLP: restriction fragment length polymorphism, NS: not significant.

Table 5 : Serum lipids levels (mmoL/L) in relation to three genotypes of signal peptide insertion/deletion polymorphism of apo B gene in the case and control Chinese geriatric subjects [Mean] [(SD)]

Subject	Genotype	n	TC	TG	LDL-C	HDL-C
Case	II	7	7.89 (0.45)	2.70 (1.72)	5.73 (0.74)	1.20 (0.37)
	ID	8	8.04 (0.96)	2.35 (1.05)	5.91 (0.96)	1.26 (0.31)
Control	II	12	5.78 #@ (1.14)	1.47 # (1.00)	3.85 #* (1.07)	1.51 #@ (0.52)
	ID	9	5.19 # (1.11)	1.27 # (0.75)	3.11 #@ (0.96)	1.51 #@ (0.60)
	DD	3	5.70 #@ (0.50)	2.79 # (0.87)	3.37 @* (1.00)	1.06 # (0.10)

* $p < 0.05$, # $p < 0.01$, @: not significant by students' t-test.

Abbreviations- n: number of samples, TC: Total Cholesterol, TG: Triglyceride, LDL-C: LDL-Cholesterol, HDL-C: HDL-Cholesterol, I: signal peptide insertion allele, D: signal peptide deletion allele.

Table 6 : Serum lipids levels (mmoL/L) in relation to two genotypes of XbaI polymorphism of apo B gene in the case and control Chinese geriatric subjects

[Mean]
[(SD)]

Subject	Genotype	n	TC	TG	LDL-C	HDL-C
Case	X-X-	15	7.97 (0.74)	2.49 (1.36)	1.23 (0.85)	5.84 (0.31)
	X-X+	0	-	-	-	-
Control	X-X-	22	5.56 (1.13)	1.52 # (1.03)	3.49 (1.08)	1.50 # (0.52)
	X-X+	2	5.40 (0.85)	2.01 # (0.30)	3.60 (0.57)	0.88 # (0.11)

# $p < 0.01$ by students' t-test.

Abbreviations- TC: Total Cholesterol, TG: Triglyceride, LDL-C: LDL-Cholesterol, HDL-C: HDL-Cholesterol, +,- referring to presence or absence of cutting site for restriction enzyme XbaI, X: XbaI.

Table 7 : Serum lipids levels (mmoL/L) in relation to two genotypes of EcoRI polymorphism of apo B gene in the case and control Chinese geriatric subjects [Mean] [(SD)]

Subject	Genotype	n	TC	TG	LDL-C	HDL-C
Case	E+E+	13	7.92 * (0.72)	5.72 * (0.97)	5.72 # (0.76)	1.29 # (0.29)
	E+E-	2	8.30 * (0.99)	7.40 * (3.37)	7.40 # (0.01)	0.88 # (0.24)
Control	E+E+	4	5.85 # (0.60)	2.37 # (1.16)	3.68 # (0.50)	1.11 # (0.14)
	E+E-	20	5.45 # (1.16)	1.36 # (0.94)	3.44 # (1.07)	1.54 # (0.58)

* $p < 0.05$, # $p < 0.01$ by students' t test.

Abbreviations- TC: Total Cholesterol, TG: Triglyceride, LDL-C: LDL-Cholesterol, HDL-C: HDL-Cholesterol, +, - referring to presence of absence of cutting site for specific restriction enzyme EcoRI, E: EcoRI.

APPENDIX I

REFERENCE INTERVAL

(Chemical Pathology Laboratory

Prince of Wales Hospital)

Total Cholesterol	< 5.2 mmoL/L	Desirable
	5.2 - 6.2 mmoL/L	Borderline High
	> 6.2 mmoL/L	High
Triglyceride	< 2.0 mmoL/L	Desirable
HDL Cholesterol	> 1.2 mmoL/L (F)	Desirable
	> 1.0 mmoL/L (M)	Desirable
LDL Cholesterol	< 3.4 mmoL/L	Desirable
	3.4 - 4.1 mmoL/L	Borderline High
	> 4.1 mmoL/L	High Risk

APPENDIX II

CALCULATION OF EXPECTED NUMBER OF SAMPLES

(Take female cord blood samples of signal peptide insertion/deletion polymorphism of apo B gene as an example)

Genotype	Number of samples	Number of allele I	D	Total number of alleles
II	41	82	-	-
ID	13	13	13	-
DD	5	-	10	-
Total	59	95	23	118

Genotype	Expected number of samples
II	38.7
ID	18.2
DD	2.1

Expected number of samples

Let x be the frequency of I allele and y be the frequency of D allele

$$x = \frac{\text{Number of I allele}}{\text{Total number of alleles}} = \frac{95}{118} = 0.81$$

$$y = \frac{\text{Number of D allele}}{\text{Total number of alleles}} = \frac{23}{118} = 0.19$$

Expected number of II genotype = x^2 (number of samples)
= $(0.81)^2$ (59)
= 38.7

Expected number of ID genotype = 2xy (number of samples)
= 2(0.81)(0.19)(59)
= 18.2

Expected number of DD genotype = y^2 (number of samples)
= $(0.19)^2$ (59)
= 2.1

6. REFERENCES

1. Helio T. The apolipoprotein B gene: its relation to coronary heart disease and serum lipid levels among the Finns. Academic dissertation, 1992.

2. Humphries SE, Dunning A, Xu CF, Peacock R, Talmud P and Hamsten A. DNA polymorphism studies - approaches to elucidating multifactorial ischaemic heart disease: the apo B gene as an example. Ann Med, 24(5): 349-356, 1992.

3. Mahley RW, Karl H, Innerarity TL and Rall SC. Genetic Defects in lipoprotein metabolism - Elevation of atherogenic lipoproteins caused by impaired catabolism. JAMA, 265: 78-83, 1991.

4. Humphries SE, Talmud P, Peacock R, Dunning A, Hamsten A and Tornvall P. Apolipoprotein B gene polymorphisms, lipoproteins and coronary atherosclerosis: A study of young myocardial infarction survivors and healthy population based individuals. Atherosclerosis, 92: 151-164, 1992.

5. Ludwig EH, Blackhart BD, Pierotti VR, Fortier C, Knott T, Scott J, Mahley RW, Levy BW and McCarthy BJ. DNA sequence of the human apolipoprotein B gene. DNA, 6: 363-372, 1987.

6. Humphries SE, Saha N, Tong MC, Tay JSH and Jeyaseelan K. DNA polymorphisms of the apolipoprotein B gene in Chinese coronary artery disease patients. Clinical Genetics, 42: 164-170, 1992.

7. Goldstein JL and Brown MS. The LDL receptor defect in familial hypercholesterolemia. Implications for pathogenesis and therapy. Med Clin North Am, 66: 335-62, 1982a.

8. Goldstein JL and Brown MS. Metabolic risk factors in ischemic cardiovascular disease: Insights into the pathogenesis of atherosclerosis derived from studies of familial hypercholesterolemia. Eds. Carlson LA and Pernow B Raven Press, New York: 17-34, 1982b.

9. Goldstein JL, Brown MS, Anderson RGW, Russell DW and Schneider WJ. Receptor mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Bio, 1:1-39, 1985.

10. Goldstein JL, Brown MS. Regulation of low density lipoprotein receptors. Implications for pathogenesis and therapy of hypercholesterolemia and atherosclerosis. Circulation, 76: 504-507, 1987.

11. Blackhart BD, Ludwig EM, Pierotti VR, Caiati L, Onasch MA, Wallis SC, Powell L, Pease R, Knott TJ, Chu ML, Mahley RW, Scott J, McCarthy BJ and Levy WB. Structure of the human apolipoprotein B gene. J Biol Chem, 261: 15364-15367, 1986.

12. Chen SH, Habib G, Yang CY, Gu ZW, Lee BR, Gotto AM, Li WH and Chan L. Apolipoprotein B-48 is the product of a messenger RNA with an organ specific in-frame stop codon. Science, 238: 363-366, 1987.

13. Yang CY, Gu ZW, Weng SA, Kim TW, Chen SH, Pownall HJ, Sharp PM, Liu SW, Li WH, Gotto AM and Chan L. Structure of apolipoprotein B-100 of human low density lipoprotein. Arteriosclerosis, 9(1): 96-108, 1989.

14. Zannis VI. Molecular biology of human apolipoproteins B and E and associated diseases of lipoprotein metabolism. Advances in lipid research, 23: 1-64, 1989.

15. Vega GL and Grundy SM. In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. J Clin Invest, 78: 1410-1414, 1986.

16. Series JJ, Gaffney D, Packard CJ and Shepherd J. Frequency of the Xba I, Eco RI, Pvu II and Msp I polymorphisms of the

apolipoprotein B gene in relation to hypercholesterolaemia in the general population. Clinical Chimica Acta, 215: 89-98, 1993.

17. Dunning AM, Tikkanen MJ, Ehnholm C, Butler R and Humphries SE. Relationships between DNA and protein polymorphisms of apolipoprotein B. Human genetics, 78: 325-329, 1988.

18. Saha N, Tay JSH, and Chew LS. Influence of apolipoprotein B signal peptide insertion/deletion polymorphism on serum lipids and apolipoproteins in a Chinese population. Clinical Genetics, 41: 152-156, 1992.

19. Visvikis S, Cambou JP, Arveiler D, Evans AE, Parra HJ, Aguillon D, Fruchart JC, and Siest G. Apolipoprotein B signal peptide polymorphism in patients with myocardial infarction and controls. Human Genetics, 90: 561-565, 1993.

20. Humphries SE, Renges HH, Wile DB, Mckeigue PM and Marmot MG. Apolipoprotein B gene polymorphisms are associated with lipid levels in men of South Asian descent. Atherosclerosis, 91: 267-275, 1991.

21. Monsalve MV, Humphries SE, Young R, Jobsis J, Dham SA, Powell S, and Greenhaigh RM. DNA polymorphism of the gene for apolipoprotein B in patients with peripheral arterial

disease. Atherosclerosis, 70: 123-129, 1988.

22. Myant NB, Humphries SE, Gallagher J, Barbir M, Thompson GR and Wile D. Restriction fragment length polymorphisms in the apo B gene in relation to coronary artery disease. Atherosclerosis, 77: 193-201, 1989.

23. Genest JJ, Ordovas JM, McNamara JR, Robbins AM, Meade T, Cohn SD, Salem DN, Wilson PWF, Masharani U, Frossard PM and Schaefer EJ. DNA polymorphisms of the apolipoprotein B gene in patients with premature coronary artery disease. Atherosclerosis, 82: 7-17, 1990.

24. Hegele RA, Huang LS, Herbert PN, Conrad BB, Buring JE, Hennekens CH and Breslow JL. Apolipoprotein B gene DNA polymorphism associated with myocardial infarction, New Engl J Med, 315: 1509-1515, 1986.

25. Randall LL, Hardy SJS. Unity in function in the absence of consensus in sequence: role of leader peptide in export. Science, 243: 1156-1159, 1989.

26. Berg K. DNA polymorphism at the apolipoprotein B locus is associated with lipoprotein level. Clin Genet, 30: 515-520, 1986.

27. Law A, Wallis SC, Powell LM, Pease RJ, Brunt H, Priestley LM, Knott TJ, Scott J, Altman DG, Miller GJ, Rajput J and Miller NE. Common DNA polymorphism within coding sequence of apolipoprotein B gene associated with altered lipid levels. Lancet, 1: 1301-1303, 1986.

28. Talmud PJ, Barni N, Kessling AM, Carsson P, Darnfors C, Bjursell G, Galton D, Wynn V, Kirk H, Hayden MR and Humphries SE. Apolipoprotein B gene variants are involved in the determination of serum cholesterol levels: a study in normo and hyperlipidaemic individuals. Atherosclerosis, 67: 81-89, 1987.

29. Aalto S, Gyling H, Helve E, Kovanen P, Miettiene TA, Turtola H and Kontula K. Genetic polymorphism of the apolipoprotein B gene locus influences serum LDL cholesterol level in familial hypercholesterolaemia. Human Genet, 82: 305-307, 1989.

30. Paulweber B, Friedl W, Krempler F, Humphries SE and Sandhofer F. Association of DNA polymorphism at the apolipoprotein B gene locus with coronary heart disease and serum very low density lipoprotein levels. Arteriosclerosis, 10: 17-24, 1990.

31. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, 2nd ed., 6.3-6.6, 14.5-14.20, 1982.

32. Henry AE. PCR technology: Principles and applications for DNA amplification. M Stockton Press. New York, USA, 7-60, 1989.

33. William JM. Illustrated Textbook of Clinical Chemistry. Gower Medical Publishing, New York, USA, 219-232, 1989.

34. Beatriz LW, Soria L, Ludwig EH, Argyres M, Brooks AR, Balckhart BD, Friedl W and McCarthy BJ. A polymorphism in a region with enhancer activity in the second intron of the human apolipoprotein B gene. Journal of Lipid Research, 32: 137-145, 1991.

35. Daly LE, Bourke GJ and McGilvray J. Interpretation and uses of medical statistics. Blackwell Scientific Publications. Fourth Edition, Oxford, UK, 102-110, 120-123, 127-130, 1991.

36. Barter PJ, Chang LBF and Rajaram OV. Sodium oleate promotes a redistribution of cholesteryl esters from high to low density lipoproteins. Atherosclerosis, 84: 13-24, 1990.

37. **Miller et al.** Genomic DNA extraction. **Nucleic acid research, 16: 1215, 1988.**